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Production of flavin mononucleotide by metabolically engineered yeast *Candida famata*

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ABSTRACT

Recombinant strains of the flavinogenic yeast *Candida famata* able to overproduce flavin mononucleotide (FMN) that contain *FMN1* gene encoding riboflavin (RF) kinase driven by the strong constitutive promoter *TEF1* (translation elongation factor 1α) were constructed. Transformation of these strains with the additional plasmid containing the *FMN1* gene under the *TEF1* promoter resulted in the 200-fold increase in the riboflavin kinase activity and 100-fold increase in FMN production as compared to the wild-type strain (last feature was found only in iron-deficient medium).

Overexpression of the *FMN1* gene in the mutant that has deregulated riboflavin biosynthesis pathway and high level of riboflavin production in iron-sufficient medium led to the 30-fold increase in the riboflavin kinase activity and 400-fold increase in FMN production of the resulted transformants. The obtained *C. famata* recombinant strains can be used for the further construction of improved FMN overproducers.

1. Introduction

Riboflavin (RF) is essential vitamin for human nutrition and animal feeding as precursor of two coenzymes: riboflavin-5′-phosphate (flavin mononucleotide (FMN)) and riboflavin-5′-adenosine diphosphate (flavin adenine dinucleotide (FAD)).

The flavin coenzymes are involved in a wide range of biochemical processes, particularly in mitochondrial electron transport, photosynthesis, fatty acid oxidation, metabolism of vitamins B_6, B_9 (folates) and B_{12}. FMN and FAD play a pivotal role in the dehydrogenation of metabolites in one- and two-electron transfer reactions, in the activation of oxygen in oxidase, oxygenase and hydroxylase reactions. Therefore flavins are widely used in medicine and are also important as biochemical reagents (Fischer and Bacher, 2005; Joosten and van Berkel, 2007; Powers, 2003). FMN has an advantage for using in food industry as yellow colorant for soft drinks and yogurt because of its much higher solubility as compared to RF (Kroschwitz et al., 1998).

In eukaryotes, RF is converted into coenzymes via the sequential actions of ATP:RF phosphotransferase (RF kinase, EC 2.7.1.26), which phosphorylates the vitamin to FMN and ATP:FMN adenylyl transferase (FAD synthetase, EC 2.7.7.2), which adenylates FMN to FAD. Both enzymes have been purified from yeast and rat tissues, and biochemically characterized (Kashchenko and Shavlovskii, 1976; McCormick et al., 1997; Schrecker and Kornberg, 1950).

In contrast to eukaryotes, most prokaryotes have a single bifunctional protein, which exhibits both RF kinase and FAD synthetase activities (Ammelburg et al., 2007; Clarebout et al., 2001; Gerdes et al., 2002; Grill et al., 2008; Hagihara et al., 1995; Mack et al., 1998; Mashhadi et al., 2008; Mayhew and Wassink, 1980; Snoswell, 1957; Solovieva et al., 1999).

The eukaryotic gene encoding a monofunctional RF kinase was firstly identified in the yeast *Saccharomyces cerevisiae* and named *FMN1* (Santos et al., 2000). This gene codes for a membrane-bound protein located in microsomes and in mitochondria. The human ortholog encodes an 18.5 kDa protein (Fmn1p), which crystal structure has been determined (Karthikeyan et al., 2003).

There are many microorganisms that can overproduce RF but they cannot overproduce flavin nucleotides (Shimizu, 2001; Sibirny et al., 2006). Only *Eremothecium ashbyii* is capable to overproduce FAD and excrete it into medium as small additive to free RF (Nagatsu et al., 1963).

Various methods were proposed for the microbial production of flavin nucleotides. As a first step to establish the method for industrial production of FAD and FMN, the bifunctional FAD synthetase gene from *Corynebacteriurn ammoniagenes* (displays

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both RF kinase and FAD synthetase activities) was cloned and overexpressed in this bacterium (Hagihara et al., 1995). Using the FAD-synthetase-overproducing recombinant cells, the FAD production from exogenous FMN (or RF) and ATP, and the specific production of FMN from RF and ATP in the presence of high concentrations of Zn^{2+} was developed (Hagihara et al., 1995). For FMN production, however, this method had the serious disadvantage as it required addition of expensive ATP and produced small amounts of FAD in addition to FMN, and separation of these nucleotides is laborious and expensive process. Later on, a method of FMN production without FAD accumulation in recombinant *C. ammoniagenes* cells was proposed (Nakagawa et al., 1995).

In a similar manner, the genetically engineered strains of *Escherichia*, *Enterobacter* or *Pseudomonas* overexpressing bifunctional flavokinase/FMN adenylyltransferase were used for obtaining the overproducers of flavin nucleotides (Efimov et al., 1998; Kitatsuji et al., 1996; Mack et al., 1998). The main drawback of all the above-mentioned methods is requirement for addition of high concentrations of expensive ATP or its precursors and RF into cultural medium for providing cells with sufficient amounts of substrates for phosphorylation reaction.

In most organisms, almost all intracellular flavins are represented by FMN and FAD. In flavinogenic yeasts (organisms capable of RF overproduction under cultivation in iron-deficient medium) RF kinase is characterized by constitutive and relatively high activity as compared to activities of the enzymes involved in RF synthesis, which are derepressed in iron-depleted conditions (Sibirny, 1996). Growth of flavinogenic yeasts in iron-deficient media results in considerable overproduction of RF but not FMN in spite activity of RF kinase is higher than that of enzymes involved in RF production under such conditions (Sibirny et al., 2006). Hydrolysis of FMN synthesized in cells by nonspecific phosphatases was hypothesized to be the possible explanation for such phenomenon (Kumar and Rao, 1968; Uhlmann et al., 1974). Among yeasts, only the flavinogenic species *C. famata* can accumulate trace amounts of FMN in cultural medium (our unpublished observations).

We showed earlier that overexpression of *FMN1* gene in the yeast *C. famata* resulted in the increase of RF kinase activity and FMN production by recombinant strains (Ishchuk et al., 2006; Voronovsky et al., 2007). However, despite the high RF kinase activity in recombinant strains, the accumulation of FMN was still low and large amounts of RF were detected in cultural medium. In this paper, we report on engineering recombinant strains with improved FMN production. This was achieved by multicopy integration of *FMN1* gene into genome of *C. famata* RF overproducing strain AF-4.

2. Materials and methods

2.1. Yeast strains, media, cultivation conditions

The yeast strains used in this study are *Debaryomyces hansenii* strain CBS 767 (The Centraalbureau voor Schimmelcultures, Utrecht, Netherlands); *Candida famata* strain VKM Y-9 (All-Russian Collection of Microorganisms, Poushchino, Russia); *C. famata* L20105 (*leu2*, NRRL Y-30292, Northern Regional Research Center, Peoria, IL, USA), it is a derivative of *C. famata* VKM Y-9 wild-type strain (Voronovsky et al., 2002); and the *C. famata* mutant AF-4 that has a deregulated RF synthesis pathway and high level of RF production (Sibirny et al., 2008). The AF-4 strain *C. famata* was obtained in our laboratory using conventional mutagenesis of *C. famata* VKM Y-9 wild-type strain and selective media. As mutagens the ultraviolet irradiation, nitrosoguanidine and ethylmethanesulfonate were used. Selection of flavinogenic strain by conventional mutagenesis includes six stages which were described in UA patent (Sibirny et al., 2008).

Yeast cells were cultivated at 30 °C in YPD medium (0.5% yeast extract, 1% peptone and 2% glucose) or modified Burkholder medium (this contained per 1 l: sucrose, 20 g; urea, 1 g; KH_2PO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 6H_2O$, 0.2 g; biotin, 1 μg. Trace salt at the final concentrations: 0.2 μM $CuSO_4$, 1.25 μM KI, 4.5 μM $MnSO_4$, 2.0 μM $NaMoO_4$, 0.75 μM H_3BO_3, 17.5 μM $ZnSO_4$).

The *Escherichia coli* strain DH5α (Φ80d*lacZ* ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*) U 169) was used as a host for selection and propagation of plasmids involved in the study. This strain was grown at 37 °C in LB medium as described previously (Sambrook et al., 1989). Transformed *E. coli* cells were maintained on a medium containing 100 mg/l of ampicillin.

If necessary 2% agar was added to solidify the media.

2.2. DNA manipulations

The sequence of *D. hansenii* CBS 767 gene encoding RF kinase was obtained from the Genolevures database (DEHA0F21186g; http://cbi.labri.fr/Genolevures/elt/DEHA). The *C. famata TEF1* promoter (translation elongation factor 1α) was isolated as described before (Ishchuk et al., 2008).

The plasmid p19L2_pr*TEF1_FMN1Dh* was constructed earlier (Ishchuk et al., 2006). The plasmid p19L2_*ble_RIB1Cf_prTEF1_FMN1Dh* (Fig. 1) was constructed according to the following description. The *FMN1* gene encoding RF kinase was amplified by PCR from the genomic DNA of *D. hansenii* CBS 767; the promoter of the *TEF1* gene was amplified by PCR from the genomic DNA of *C. famata* (VKM Y-9). Designed primers for the *FMN1* gene included sites for *Sal*I (underlined): the forward primer (5′-CCC GTC GAC ATG ACG AGA CCT GAA ACT CAC GTT CC-3′), the reverse primer (5′-CGG GTC GAC CCT TAA GTA AAA GTA CCC CAA AAT AGA AAC G-3′). Designed primers for the *TEF1* gene promoter included sites for *Bam*HI and *Sal*I, respectively (underlined): the forward primer (5′-TGC GGA TCC TAA CGA ACA GCT CAT CAG-3′), the reverse primer (5′-TGC GTC GAC TTT GCT TAA TGT ATA ATA ATA G-3′). Expected fragments of 824 bp (the *FMN1* gene) and 658 bp (the *TEF1* promoter) were isolated. The *Bam*HI-*Sal*I-fragment with *C. famata TEF1* promoter and *Sal*I-fragment with *FMN1* ORF of *D. hansenii* were cloned into the plasmid pTCfRIB1 (5.8 kb) (Dmytruk et al., 2006). The resulting plasmid was designated p19L2_*ble_RIB1Cf*_pr*TEF1_FMN1Dh*.

Plasmid and genomic DNA isolation, DNA restriction and ligation, agarose gel electrophoresis, Southern blot analysis of yeast genomic DNA were carried out as described in Sambrook et al. (1989).

E. coli transformations were performed by electroporation (Sambrook et al., 1989); *C. famata* electrotransformations were

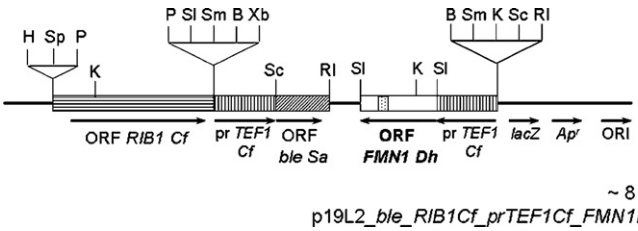

Fig. 1. The linear scheme of the plasmid p19L2_ble_*RIB1Cf_prTEF1_FMN1Dh* (~8 kb). The construct contains a whole sequence of pUC19 (it is shown as thin line); *C. famata* DNA fragment with *RIB1* gene, box with horizontal hatches; *C. famata* DNA fragment with *TEF1* promoter, box with vertical hatches; bacterial *ble* gene, box with slanting hatches; *D. hansenii FMN1* gene with intron, open box. Restriction sites: H, *Hind*III; Sp, *Sph*I; P, *Pst*I; K, *Kpn*I; RI, *Eco*RI; Sl, *Sal*I; B, *Bam*HI; Sm, *Sma*I; Sc, *Sac*I; Xb, *Xba*I.